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## Decreased telomere length in children with cartilage-hair hypoplasia

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**TITLE PAGE**

**Decreased telomere length in children with cartilage-hair hypoplasia.**

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22 **ABSTRACT**

23

24 **Background:** Cartilage-hair hypoplasia (CHH) is an autosomal recessive chondrodysplasia caused by *RMRP*  
25 (RNA component of mitochondrial RNA processing endoribonuclease) gene mutations. Manifestations  
26 include short stature, variable immunodeficiency, anemia and increased risk of malignancies, all of which  
27 have been described also in telomere biology disorders. *RMRP* interacts with the telomerase reverse  
28 transcriptase (TERT) subunit, but the influence of *RMRP* mutations on telomere length is unknown. We  
29 measured relative telomere length (RTL) in patients with CHH, their first-degree relatives and healthy  
30 controls, and correlated RTL with clinical and laboratory features.

31 **Methods:** The study cohort included 48 CHH patients with homozygous (n=36) or compound heterozygous  
32 *RMRP* mutations (median age 38.2 years, range 6.0-70.8 years), 86 relatives (74 with a heterozygous *RMRP*  
33 mutation) and 94 unrelated healthy controls. We extracted DNA from peripheral blood, sequenced the *RMRP*  
34 gene and measured RTL by quantitative-PCR.

35 **Results:** Compared with age- and sex-matched healthy controls, median RTL was significantly shorter in  
36 CHH patients (n=40 pairs, 1.05 vs 1.21, p=0.017), but not in mutation carriers (n=48 pairs, 1.16 vs 1.10,  
37 p=0.224). RTL correlated significantly with age in *RMRP* mutation carriers (rho -0.482, p<0.001) and non-  
38 carriers (rho -0.498, p<0.001), but not in patients (rho -0.236, p=0.107). Especially children (<18 years) with  
39 CHH had shorter telomeres than controls (median RTL 1.12 vs 1.26, p=0.008). In patients with CHH, RTL  
40 showed no correlation with genotype, clinical or laboratory characteristics.

41 **Conclusions:** Telomere length was decreased in children with CHH. We found no correlation between RTL  
42 and clinical or laboratory parameters.

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46 **KEY WORDS**

47

48 Bone marrow failure, relative telomere length, *RMRP*, telomerase, telomere biology disorders.

49    **INTRODUCTION**

50

51            Telomeres constitute the protective end-parts of human chromosomes and can contribute to the  
52 pathogenesis of aging and cancer. The phenotype of inherited telomere disorders includes bone marrow  
53 failure, malignancies, pulmonary fibrosis, liver cirrhosis, diabetes, and cardiovascular and gastrointestinal  
54 diseases [1]. Telomeres shorten with every cell division, but the loss can be compensated by telomerase-  
55 mediated elongation. The telomerase ribonucleoprotein complex consists of an RNA template, a catalytic  
56 reverse transcriptase subunit (TERT) and associated proteins that affect assembly, stability and recruitment of  
57 telomerase to telomeres (e.g. DKC, NOP10, NHP2, and GAR). In addition to telomere elongation,  
58 telomerase has also been associated with regulation of a number of cellular functions including survival,  
59 inflammation, apoptosis, transcription and metabolism [2].

60            Cartilage-hair hypoplasia (CHH, MIM #250250) is a rare autosomal recessive metaphyseal  
61 chondrodysplasia caused by biallelic mutations in the *RMRP* (RNA component of mitochondrial RNA  
62 processing endoribonuclease) gene [3]. The disease is over-represented in the Amish and Finnish populations  
63 [4,5]. Clinical features include severe disproportionate short stature, hair hypoplasia, variable  
64 immunodeficiency, anemia and increased risk of malignancies. Genotype-phenotype correlations are  
65 inconsistent and clinical manifestations vary even between siblings [6]. Mutation carriers remain  
66 asymptomatic [5,7].

67            Complex pathogenesis of CHH involves cell cycle impairment and altered regulation of genes  
68 associated with cell proliferation and differentiation [8,9]. In addition, formation of a ribonucleoprotein  
69 complex by *RMRP* and TERT has been confirmed [10]. This complex produces double-stranded RNAs and  
70 regulates *RMRP* expression.

71            Disorders of telomere maintenance, like dyskeratosis congenita (DC), share some clinical features  
72 with CHH, e.g. growth retardation, bone marrow failure leading to anemia and immunodeficiency, and  
73 increased incidence of malignancies [11]. Pulmonary fibrosis has also been linked to *TERT* mutations, both  
74 in idiopathic cases and in patients with DC [12]. Interestingly, we have recently reported fibrosis-like  
75 changes on high-resolution computed tomography of the lungs in patients with CHH [13].

76            Despite the known association between *RMRP* and TERT, it remains unknown whether *RMRP*

77 mutations have a significant impact on the telomere elongating functions of telomerase. In order to further  
78 elucidate the pleiotropic consequences of *RMRP* mutations and the pathogenesis of CHH we evaluated  
79 relative telomere lengths (RTL) in a large cohort of Finnish children and adults with CHH, their first-degree  
80 relatives most of whom were heterozygous mutation carriers, and healthy controls. We also analyzed the  
81 correlation of RTL with the patients' clinical and laboratory features.

82

83 **METHODS**

84

85 Patients with genetically confirmed CHH were identified from the Finnish Chondrodysplasia  
86 Register and recruited to a study exploring clinical, genetic and immunological characteristics of CHH. Their  
87 first-degree relatives were contacted via the index persons. All individuals who agreed to participate, or their  
88 guardians, signed an informed consent. The study was approved by the Research Ethics Committee at  
89 Helsinki University Hospital, Finland.

90 Clinical and laboratory data were retrieved retrospectively from the patient hospital records, as well  
91 as prospectively during study visits. Apart from CHH-related clinical features we evaluated also factors that  
92 could theoretically influence telomere length, such as smoking, obesity (body mass index Z-score) and  
93 hormone or immunosuppressive therapy. We also selected clinical features that could emerge from telomere  
94 impairment (immunodeficiency, malignancies, short stature), as well as the need for repeated blood  
95 transfusions and immunoglobulin replacement therapy. Since growth failure in CHH is progressive, we used  
96 age- and sex-specific growth data for CHH [14] to classify patients as having mild, moderate or severe  
97 growth failure, as described previously [15]. In the majority of patients, blood samples were drawn  
98 simultaneously for telomere measurement and for analysis of immunologic parameters characterizing the  
99 degree of bone marrow deficiency and/or immunodeficiency: hemoglobin, red blood cells, leukocytes,  
100 neutrophils, lymphocytes, CD3+, CD4+, CD8+, CD19+, CD16/56+ cell counts, immunoglobulin A, M and  
101 G levels, as well as Epstein-Barr virus (EBV) viral load and antibodies.

102 **Control group.** First-degree relatives of the patients (n=86), all without features of CHH, included  
103 37 parents, 38 siblings and 11 children. Altogether, 74 of them were confirmed to be heterozygous *RMRP*  
104 mutation carriers while 12 were negative for *RMRP* mutations. The control group consisted of *RMRP*  
105 mutation-negative individuals: 1) siblings of the patients (n=12) and 2) individuals who had participated in  
106 our previous studies involving healthy children and adults (n=94). The data available on controls included  
107 age, sex, ethnic background (all of Finnish origin) and overall health (all healthy). Parts of the statistical  
108 analyses were performed in a case-control setting, and age- and sex-matched controls were selected from the  
109 control group for each patient or mutation carrier aiming at age difference of no more than 12 months.

110 **DNA extraction and *RMRP* sequencing.** Peripheral blood samples were collected for all study  
111 participants. DNA was extracted with 5 Prime Archive Pure DNA Blood kit according manufacturer's

112 instructions (5 Prime GmbH, Hilden; Germany). All samples were sequenced for *RMRP* to confirm the  
113 genotype in patients with CHH and to confirm or exclude heterozygous mutations in the patients' unaffected  
114 relatives and the healthy controls. For patients and their relatives, blood samples were collected  
115 prospectively, whereas readily available DNA (extracted with the same methods) was used for healthy  
116 controls. Primers for *RMRP* (GRCh37/hg19) were designed with Primer3 v.0.4.0  
117 (<http://frodo.wi.mit.edu/primer3/>) for the gene, with a minimum of 60 bases of flanking regions adjacent to  
118 the coding region. PCR amplification was performed with DreamTaq (ThermoScientific, Waltham, MA,  
119 USA). The DNA fragments were then visualized with Midon Green Advanced DNA Stain (NIPPON  
120 Genetics, GmbH, Europe) on a 1.2% agarose gel, purified with ExoSAP (USB, Cleveland, OH, USA) and  
121 labeled with BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). After bidirectional  
122 sequencing with an ABI3730 sequencer (Applied Biosystems), chromatograms were analyzed with  
123 Sequencher v5.0 (Gene Codes Corporation, Ann Arbor, MI, USA) using genomic NG\_017041.1 and RNA  
124 reference sequence NR\_003051.3. Primer sequences and detailed PCR protocols are available upon request.

125 **Telomere length measurement.** Telomere length was determined by the quantitative-PCR method  
126 described by Cawthon [16], with minor modifications [17]. Briefly, sample DNA was analyzed in triplicate  
127 wells in a separate Telomere (TEL) and a single copy gene (HBG) reaction on the ABI7900HT instrument  
128 (Applied Biosystems), at two separate times. TEL/HBG (T/S) values were calculated by the  $2^{-\Delta Ct}$  method,  
129 where  $\Delta Ct = \text{average } Ct_{\text{TEL}} - \text{average } Ct_{\text{HBG}}$ . RTL were generated by dividing samples T/S value with the  
130 T/S value of a reference CCRF-CEM cell line DNA included in all runs.

131 **Statistical analysis.** Subject's age on the day of blood sampling was used for the analyses. To  
132 evaluate RTL correlations, we classified individuals to categories of short, average or long RTL for age and  
133 used both, RTL itself and RTL category, in the analysis. These categories were determined based on RTL and  
134 age data from the healthy controls. Individuals with  $RTL > 0.5$  standard deviation (SD) from the regression  
135 line for age vs RTL were classified as having long RTL, those with  $RTL < -0.5$  SD as having short RTL, and  
136 the remaining as having average RTL (*Supplementary Figure 1*). We applied the Mann-Whitney test for  
137 categorical and the Spearman's coefficient (rho) for continuous variables. Linear or logistic regression  
138 analysis was used for multivariate models. A p value  $< 0.05$  was considered significant. Statistical analyses  
139 were performed with the IBM SPSS software.

## 140 RESULTS

141

142 **Patient characteristics.** Altogether, 48 Finnish patients (31 females, 17 males) with CHH  
143 participated in the study. Their median age was 38.2 years (range 6.0-70.8 years). Sanger sequencing of the  
144 *RMRP* gene showed that most of the patients (75%, 36/48) were homozygous for the g.70A>G mutation  
145 (rs199476103, now referred to as g.71A>G) while 12 patients (25%) were compound heterozygous for  
146 g.70A>G and either g.262G>T mutation (rs727502774, now referred to as g.263G>T) (n=11) or a 10-  
147 nucleotide duplication at position -13 (TACTCTGTGA, rs727502775) (n=1).

148 *Tables 1 and 2* present patients' clinical and laboratory data. Nine patients had been diagnosed with  
149 malignancies, including basal cell carcinoma (n=6), B-cell lymphoma (n=2), uterus carcinoma (n=1) and  
150 vocal cord carcinoma (n=1). In the two patients who had survived lymphoma, no data were available on  
151 EBV status in lymphoma samples. No EBV-associated diseases were reported in our patients. Blood EBV  
152 viral load was undetectable by PCR in 10 individuals and 34/41 (83%) patients tested positive for serum  
153 antibodies to EBV. Two patients had required repeated red blood cell transfusions for anemia and another  
154 four patients had been treated with immunoglobulin replacement therapy. Some individuals reported history  
155 of smoking (n=9), intake of inhaled corticosteroids for physician-diagnosed asthma (n=15) or growth  
156 hormone treatment in childhood (n=3).

157



158 *Table 1. Clinical features of 47 study subjects with cartilage-hair hypoplasia.*

Clinical feature		Number of patients	Proportion of patients
Growth deficiency			
	Severe	3/47	6%
	Moderate	17/47	36%
	Mild	27/47	58%
History of			
	Pneumonia	10/47	19%
	Rhinosinusitis	27/47	57%
	Otitis media	34/47	72%
	Warts	15/47	32%
	Hospitalization for varicella	5/34	15%
	Smoking	9/47	19%
Normal susceptibility to infections*		15/47	32%
Combined immunodeficiency**		25/47	53%
History of			
	Malignancies	9/47	19%
	Repeated blood transfusions	2/47	4%
	Immunoglobulin therapy	4/47	8%
	Immunosuppressive therapy	2/47	4%
	Growth hormone treatment	3/47	6%
	Therapy with inhaled glucocorticoids	15/47	32%
Bronchiectasis		8/30	27%
Fibrosis-like lung changes		5/30	17%

159 \* Normal susceptibility to infections was defined as occasional uncomplicated RTI or otitis media/rhinosinusitis not  
160 requiring surgical interventions, absence of pneumonias and sepsis and varicella not requiring hospitalization.

161 \*\* Patients with warts, recurrent HSV infections, varicella requiring hospitalization or malignancy were considered to  
162 have combined immunodeficiency.  
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166 *Table 2. Laboratory characteristics of study subjects with cartilage-hair hypoplasia. Cell counts are*  
167 *shown in cells x10<sup>9</sup>/l with the exception of red blood cells (x10<sup>12</sup>/l). Hemoglobin and immunoglobulin levels are in g/l.*  
168 *Normal values in adults represent local laboratory reference values.*  
169

Laboratory parameter	Normal values in adults	Patients tested (n)	Results, median (range)	Patients with decreased counts/levels, n (%)
Red blood cells	F 3.90-5.20, M 4.25-5.70	47	4.32 (2.69-5.34)	5 (11%)
Hemoglobin	F 117-155, M 134-167	47	136 (102-161)	3 (6%)
White blood cells	3.4-8.2	47	6.0 (1.2-12.0)	4 (9%)
Absolute neutrophil count	1.5-6.7	47	1.76 (0.28-8.40)	3 (6%)
Absolute lymphocyte count	1.3-3.6	47	1.31 (0.26-3.25)	26 (55%)
CD3+ cells	0.85-2.28	46	0.93 (0.16-3.01)	19 (41%)
CD4+ cells	0.458-1.406	46	0.548 (0.118-1.312)	17 (37%)
CD8+ cells	0.24-0.98	46	0.30 (0.04-1.72)	19 (41%)
CD19+ cells	0.12-0.43	46	0.12 (0.00-0.34)	30 (65%)
CD16/56+ cells	0.08-0.57	46	0.18 (0.05-0.55)	3 (7%)
Immunoglobulin A	0.52-4.84	45	1.9 (0.4-7.5)	4 (9%)
Immunoglobulin M	0.36-2.84	46	0.9 (0.2-3.1)	4 (9%)
Immunoglobulin G	6.8-15.0	44	10.7 (4.2-15.7)	1 (2%)

170 F females, M males, n number  
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**Telomere length.** RTL measurement was performed by quantitative PCR on altogether 228 samples from patients (n=48), first-degree relatives (n=86) and healthy unrelated controls (n=94). Sanger sequencing detected 74 carriers for *RMRP* mutations among the CHH patients' unaffected relatives (86%). *Table 3* demonstrates the characteristics of the participants according to the *RMRP* mutation status. There was a significant negative correlation between RTL and age in mutation carriers ( $\rho$  -0.482,  $p < 0.001$ ) and non-carriers ( $\rho$  -0.498,  $p < 0.001$ ), but not in patients ( $\rho$  -0.236,  $p = 0.107$ ) (*Figure 1*). RTL was not influenced by sex in any age group.

**Table 3.** Characteristics of patients with cartilage-hair hypoplasia, asymptomatic *RMRP* mutation carriers and non-carriers.

	<b>Patients with CHH</b>	<b><i>RMRP</i> mutation carriers</b>	<b>Mutation-negative individuals</b>
<b>Size of group (n)</b>	48	74	106
<b>Sex, F/M (%)</b>	31/17 (65%/35%)	39/35 (53%/47%)	64/42 (60%/40%)
<b>Median age, years</b>	38.2 (6.0-70.8)	48.8 (5.0-70.8)	37.1 (6.0-70.8)
<b>Number of subjects aged:</b>			
<b>5.0-18.0 years</b>	9 (19%)	17 (23%)	19 (18%)
<b>18.1-40.0 years</b>	17 (35%)	12 (16%)	40 (38%)
<b>40.1-70.8 years</b>	22 (46%)	45 (61%)	47 (44%)
<b>Mutation type (n, %):</b>			n/a
<b>homozygote, g.70A&gt;G</b>	36 (75%)	none	
<b>compound heterozygote</b>	12 (25%)	none	
<b>heterozygote, g.70&gt;G</b>	none	58 (78%)	
<b>heterozygote, other*</b>	none	16 (22%)	
<b>Relation to patients (n, %):</b>	n/a		
<b>parents</b>		37 (50%)	none
<b>siblings</b>		26 (35%)	12 (11%)
<b>children</b>		11 (15%)	none
<b>unrelated</b>		none	94 (89%)

CHH cartilage-hair hypoplasia, F female, M male, n number of subjects, n/a not applicable, *RMRP* RNA component of mitochondrial RNA processing endoribonuclease

\* other mutations included g.262G>T or a 10-nucleotide duplication at position -13 (TACTCTGTGA).

**RTL in patients and mutation carriers.** The proportion of CHH patients with short RTL for age was significantly higher (52%, 25/48) than among mutation carriers (20%, 15/74,  $p<0.001$ ) or healthy non-carriers (29%, 31/106,  $p=0.011$ ) (*Table 4*). In the sub-analysis by age group, almost all children with CHH had short telomeres for age (89%, 8/9). Further, two thirds of patients aged 18.1-40.0 years (65%, 11/17) and one fourth of those aged over 40.1 years (27%, 6/22) had short telomeres. Compared with *RMRP* mutation-negative individuals, the proportion of patients with short RTL was significantly higher in children ( $p=0.016$ ) and adults up to 40.0 years of age ( $p=0.047$ ), but not in older individuals ( $p=0.769$ ) (*Table 4*).

**Table 4.** Relative telomere length as median (range) and as short, average or long for age in various age groups of patients with cartilage-hair hypoplasia, *RMRP* mutation carriers and non-carriers. Higher proportion of patients demonstrated RTL short for age (bold) compared with mutation carriers ( $p<0.001$ ) or healthy non-carriers ( $p=0.011$ ). Compared with *RMRP* mutation-negative individuals, the proportion of patients with short RTL was significantly higher in children ( $p=0.016$ ) and adults up to 40.0 years of age ( $p=0.047$ ), but not in older individuals ( $p=0.769$ ).

Study group	Age group (years)	Number of subjects	Median RTL (range)	Subjects with short RTL, n (%)	Subjects with average RTL, n (%)	Subjects with long RTL, n (%)
Patients	All ages	48	1.07 (0.70-1.81)	<b>25 (52%)</b>	11 (23%)	12 (25%)
	6.0-18.0	9	1.12 (0.88-1.31)	<b>8 (89%)</b>	1 (11%)	0 (0%)
	18.1-40.0	17	1.08 (0.91-1.72)	<b>11 (65%)</b>	2 (11.5%)	4 (23.5%)
	40.1-70.8	22	1.06 (0.70-1.81)	6 (27%)	8 (36.5%)	8 (36.5%)
<i>RMRP</i> mutation carriers	All ages	74	1.16 (0.88-1.78)	15 (20%)	32 (43%)	27 (37%)
	5.0-18.0	17	1.30 (0.95-1.77)	7 (41%)	6 (35%)	4 (24%)
	18.1-40.0	12	1.20 (0.99-1.78)	3 (25%)	5 (42%)	4 (33%)
	40.1-70.8	45	1.09 (0.88-1.45)	5 (11%)	21 (47%)	19 (42%)
<i>RMRP</i> mutation-negative subjects	All ages	106	1.16 (0.71-2.05)	31 (29%)	45 (43%)	30 (28%)
	6.0-18.0	19	1.25 (0.86-2.05)	7 (37%)	5 (26%)	7 (37%)
	18.1-40.0	40	1.22 (0.88-1.78)	14 (35%)	17 (43%)	9 (22%)
	40.1-70.8	47	1.07 (0.71-1.45)	11 (23%)	22 (47%)	14 (30%)

n number, *RMRP* RNA component of mitochondrial RNA processing endoribonuclease, RTL relative telomere length.

We were able to find from the control group age- and sex-matched controls with no *RMRP* mutations for 40 patients (12 males, 28 females, median age 37.5 years) for case-control analyses. RTL was significantly shorter in patients (median RTL 1.05) compared with the controls (median RTL 1.21,  $p=0.017$ ). Children aged  $\leq 18$  years accounted for this difference in RTL (median 1.09 in patients vs 1.25 in controls,  $p=0.015$ ,  $n=8$  pairs), while only a trend for shorter RTL was observed in those aged  $>18$  and  $\leq 40$  years (median 1.07 vs 1.26 in controls,  $p=0.069$ ,  $n=14$  pairs) and no difference was detected in the age group  $>40$  years (median RTL 1.03 vs 1.08 in controls,  $p=0.443$ ,  $n=18$  pairs) (*Figure 2*).

When all study samples were included in analyses, a significant difference in RTL between patients with CHH and healthy subjects (including *RMRP* mutation carriers and non-carriers) was also observed in children (6.0-18.0 years of age, median RTL 1.12 in nine patients vs 1.26 in 36 controls,  $p=0.008$ ) (*Figure 3*).

220 No significant difference was detected in the older age-groups, although young adults with CHH  
221 demonstrated a tendency for shorter telomeres (median RTL 1.08 in 17 patients vs 1.22 in 52 controls,  
222  $p=0.082$ ).

223 We compared RTL in 48 *RMRP* mutation carriers with age- and sex-matched non-carrier controls,  
224 and observed no difference in the median RTL between these two groups (1.16 vs 1.10,  $p=0.224$ ) (data not  
225 shown).

226 **RTL and CHH-related characteristics and morbidity.** In the patient cohort, RTL and classified  
227 RTL (short, average or long for age) showed no correlation with the type of *RMRP* mutation, sex, history of  
228 blood transfusions, immunoglobulin substitution, hormone or immunosuppressive therapy, obesity or history  
229 of smoking. Various infectious manifestations separately or in combinations, fibrosis-like lung changes,  
230 history of malignancies, the severity of growth failure and analyzed laboratory parameters did not correlate  
231 with RTL itself or RTL category (*Supplementary Table 1*). In two patients who had survived lymphoma, RTL  
232 was average and long for age respectively.

## 233 **DISCUSSION**

234

235 Our study demonstrates shorter telomeres in DNA from peripheral blood in children with CHH. The  
236 interpretation of our findings necessitates further studies to determine whether shorter telomeres are the  
237 cause or the consequence in CHH pathology. Decreased telomere length may reflect the increased number of  
238 cell divisions required to compensate for the impaired cell cycle and increased apoptosis reported in CHH  
239 [18]. Alternatively, telomerase defects can represent primary pathologic mechanism contributing to stem cell  
240 exhaustion, including bone marrow failure present in some patients with CHH. Our findings confirm the  
241 significance of *RMRP* in telomerase function.

242 RTL correlated significantly with age in *RMRP* mutation carriers and non-carriers, but not in patients  
243 with CHH, which can be explained by shorter telomeres in pediatric patients. Individuals with CHH have  
244 increased mortality in childhood and young adulthood from infections and malignancies [19,20]. A high  
245 proportion of our patients (46%, 22/48) were over 40 years of age and thus represent a selected population of  
246 less affected patients who escaped fatal complications in early life. Therefore, normal telomere length in  
247 adults with CHH can indicate a survival advantage. The absence of correlations between telomere length and  
248 clinical and laboratory characteristics can derive from the small sample size. Also, RTL is a rough estimate of  
249 telomere structure and represents only a part of the complex telomere biology.

250 Interestingly, some DC mutation carriers demonstrate telomere shortening while remaining  
251 asymptomatic and inheritance of shorter telomeres probably induces more severe disease in subsequent  
252 generations [21]. The finding of normal telomeres in *RMRP* mutation carriers contradicts the data from  
253 families with DC. More research is needed to test pedigrees where CHH has been diagnosed in more than  
254 one generation. If the shorter telomere length is indeed inherited, this may result in more severe disease in  
255 the affected offspring of patients with CHH in future generations.

256 Cell immortalization and subsequent development of cancer can emerge from abnormal telomere  
257 maintenance [22]. While DC and CHH are both characterized by increased risk of malignancies, the types of  
258 tumors developing in patients with these disorders differ. The most common malignancies in individuals with  
259 DC include head and neck squamous cell carcinomas, while in subjects with CHH, non-Hodgkin lymphomas  
260 and basal cell carcinomas predominate [20,23]. Accordingly, in the light of present data, immunodeficiency

rather than the telomere length is the nominator in predisposition to malignancy in CHH patients. Immunodeficiency in patients with CHH may predispose them to EBV-associated lymphoproliferative disorders. Unfortunately, no data were available on EBV status in lymphoma samples from the two patients in our cohort. The majority of our patients had detectable serum antibodies to EBV but EBV DNA was not detected by PCR in peripheral blood. EBV causes telomere dysfunction in the infected cells [24, 25] and it is possible that this is relevant in CHH. Therefore, further research on alteration in telomere functions and the role of EBV in CHH patients with malignancies would be warranted.

The results of our study have important clinical implications. Patients with CHH can require hematopoietic stem cell transplantation and defective telomere biology should be taken into account when choosing conditioning regimen. Also, bone marrow failure in patients with DC has been successfully treated with androgens (probably due to telomerase up-regulation) and this treatment option may be considered in selected patients with CHH [26-28]. Furthermore, telomere length measurement may guide diagnostic process in individuals with immunodeficiency of unknown etiology and CHH should be included in the differential diagnosis of children with short telomeres.

We recognize strengths and limitations in our study. This is the first study to evaluate telomere length in patients with CHH. The high prevalence of the disease in our population provided us with a unique opportunity to recruit a large cohort of patients and their unaffected relatives with a homogenous genetic and ethnic background and with a wide age range; in rare diseases such an approach is seldom possible. The drawbacks of our study include insufficient clinical data from relatives of patients with CHH and healthy controls. Thus, it was impossible to analyze the influence of e.g. smoking or medications on RTL. However, in the patient group, these factors did not affect telomere length and none of the patients aged <18 years reported smoking. The number of healthy controls was rather small, which increases the risk of bias and due to the inter-individual variability of telomere length our data may not be applicable to particular individuals with CHH. Also, no data were available on metabolic profile (glucose, insulin and lipid profile, blood pressure) of our patients, hindering the evaluation of its relationship with RTL. Another limitation is the use of RTL, where mean telomere length of all chromosome ends is estimated. Telomere length distributions and critically short individual telomere ends cannot be detected by this method.

Our results suggest that telomere length is abnormal in children with CHH. Further studies are required to explore functional consequences of altered telomere maintenance and possible clinical

290 implications of these findings. Longitudinal follow-up of patients with CHH is necessary to establish the  
291 significance of telomere length as a predictor of disease severity and mortality.  
292



293 **CONTRIBUTORSHIP STATEMENT**

294

295 Study design: PK, MT, GR and OM. Study conduct: SK, MP, SD. Data collection: SK. Data analysis: SK,

296 SD, ML. Data interpretation: SK, SD, MT, GR, OM. Drafting manuscript: SK. Revising manuscript content:

297 All authors. Approving final version of manuscript: All authors.

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306     **COMPETING INTERESTS**

307

308     The authors declare no conflicts of interest.

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310

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313    measurements.

314

315     **CONFLICT OF INTEREST STATEMENT**

316

317     The authors declare no conflicts of interest.

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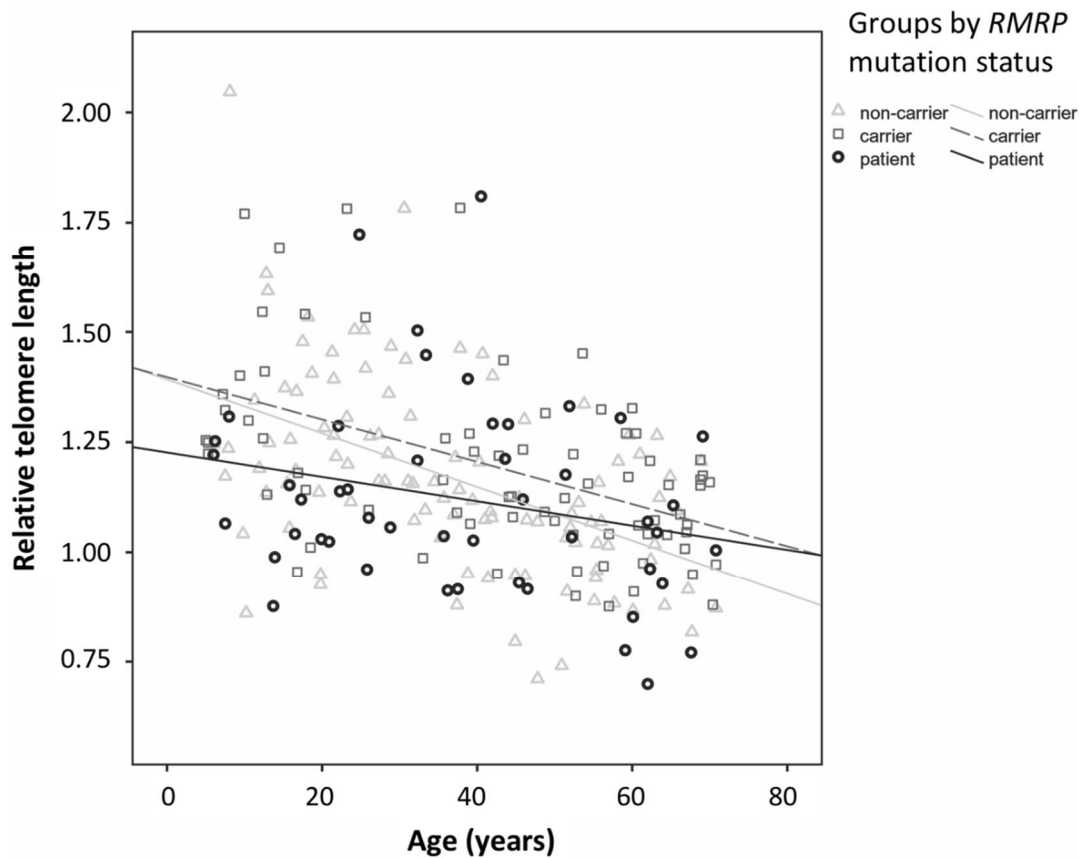


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417 **LEGENDS TO FIGURES**

418

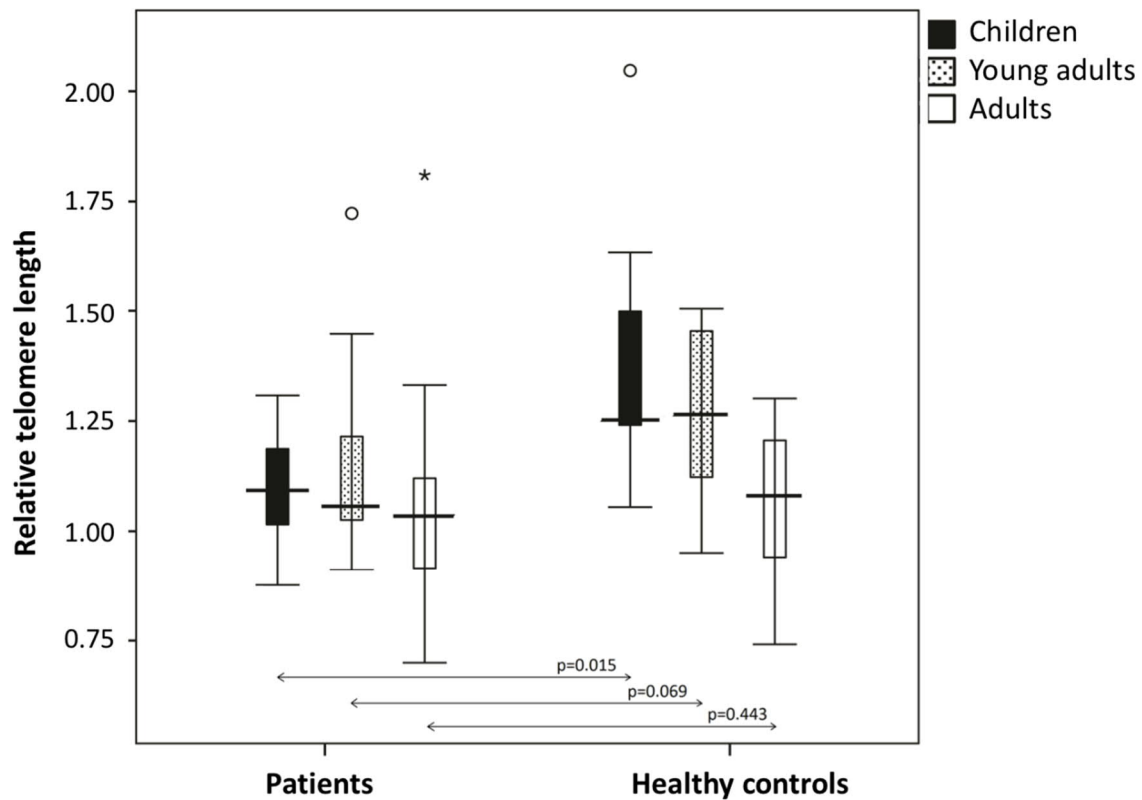
419 *Figure 1.* Correlation of relative telomere length with age in 228 samples from patients with cartilage-hair  
420 hypoplasia, their relatives and healthy controls. Every dot corresponds to a measurement from a single  
421 individual. RTL correlated significantly with age in *RMRP* mutation carriers (rho -0.482, p<0.001) and non-  
422 carriers (rho -0.498, p<0.001), but not in patients (rho -0.236, p=0.107).



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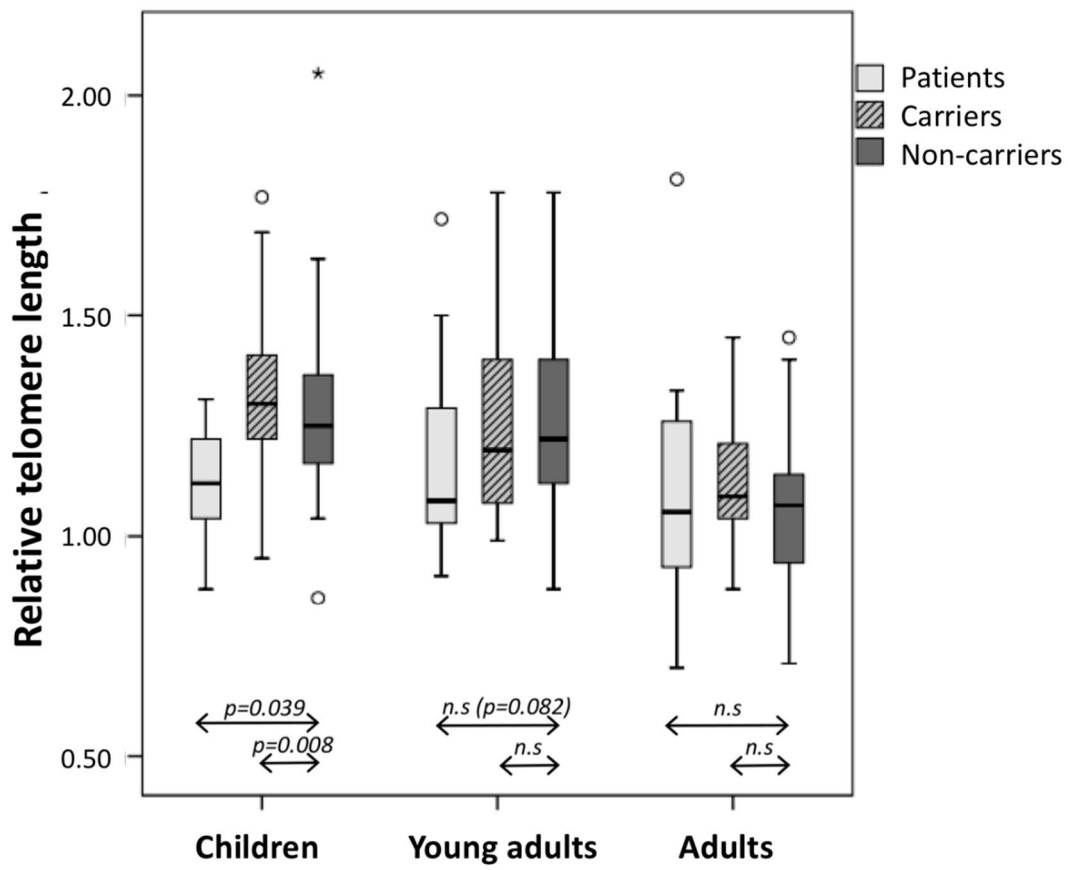
425 *Figure 2.* Comparison of relative telomere length (RTL) in a case-control setting in 40 patients with  
426 cartilage-hair hypoplasia (median RTL 1.05) and 40 age- and gender-matched healthy controls negative for  
427 *RMRP* mutation (median RTL 1.21, p=0.017). RTL was significantly shorter in children with CHH (median  
428 1.09 vs 1.25 in controls, p=0.015, n=8 pairs). Adults with CHH showed a trend for shorter RTL in the age  
429 group >18 and ≤40 years (median 1.07 vs 1.26 in controls, p=0.069, n=14 pairs), while no difference was  
430 detected in the age group >40 years (median RTL 1.03 vs 1.08 in controls, p=0.443, n=18 pairs).



431

432

433 *Figure 3.* Comparison of relative telomere length (RTL) in different age groups of patients with cartilage-hair  
 434 hypoplasia (CHH), *RMRP* mutation carriers and non-carriers. Children with CHH (aged  $\leq 18$  years)  
 435 demonstrate significantly shorter RTL compared with asymptomatic mutation carriers and non-carriers. No  
 436 difference in RTL was observed in young adults (aged  $>18$  and  $\leq 40$  years) or adults (aged  $>40$  years) with  
 437 CHH compared with controls.



438

439

440	<b>ABBREVIATIONS</b>
441	
442	CHH, cartilage-hair hypoplasia
443	DC, dyskeratosis congenita
444	EBV Epstein-Barr virus
445	rho, Spearman's correlation coefficient
446	<i>RMRP</i> , RNA component of mitochondrial RNA processing endoribonuclease
447	RTL, relative telomere length
448	SD standard deviation
449	TERT, telomerase reverse transcriptase